An Ecological Factor of Significance in the Study of Bacterial Populations of the Human Small Intestine Using Ileostomy Specimens [1].

by

Pasquale V. Scarpino [2].

and

Richard W. Niemeier [3].

Presented at the Joint Meeting

of the

Indiana and Ohio Branches

of the

American Society for Microbiology

Held at

Hueston Woods Lodge

Oxford, Ohio

November 14 - 15, 1969

1. This project was supported by funds granted to the principal investigator from the National Aeronautics and Space Administration's Multidisciplinary Grant with the Institute of Space Sciences of the University of Cincinnati.

2. Associate Professor of Environmental Health Engineering (Microbiology), Department of Civil Engineering, University of Cincinnati.

3. Former graduate student in Environmental Health Engineering (Microbiology), Department of Civil Engineering, University of Cincinnati.

Baseline investigations of the aerobic and anaerobic microbes that inhibit the intestinal tract of man are necessary for proper evaluation of the environmental effects of space on the intestinal flora. Previous studies by us (Scarpino, Deters, and Niemeier, 1969) indicated a relative stability in the microflora found in ileostomy subjects, and it was suggested at the time that the presence or absence of bacteriostatic substances would account for the stability of this microflora. Previous investigations shown in the first Table detected a bacteriostatic substance or substances in specimens obtained from the human terminal ileum of one subject that inhibited 10 of 18 pure cultures of intestinal bacteria. Most bacteria normally occurring in the ileum were not inhibited, whereas the more uncommon types were inhibited. These results were considered to account, at least in part, for the stability of types and numbers of organisms noted in the terminal ileum.

Six ileostomy subjects were subsequently studied to determine the presence of such bacteriostatic substances in specimens obtained from the terminal end of their ileums. Only one subject, number five, a 35 year old man with a 10 year ileostomy, demonstrated dramatic evidence of its presence, and the results of our studies with subject number five will be reported here. The other subjects exhibited either a small degree of inhibitory activity against indicator strains, or no activity at all. Forty pure cultures that were predominantly fecal in origin were used initially as indicator strains for this substance, but later the indicator strains were limited to two of Escherichia coli, and one of Shigella sonnei.

Zones of stimulation, i.e. heavier growth, also were noted periodically with most of the extracts obtained. Stimulation had no apparent relation to indicator organism inhibition. Thus, when some indicator organisms were inhibited, they sometimes developed heavier growth, i.e. zones of stimulation, outside the perimeter of the zone of inhibition. At other times no inhibition was noted but stimulation occurred.

TABLE 1

THE INFLUENCE OF A BACTERIA-FREE FILTRATE FROM THE HUMAN ILEUM ON 18 PURE CULTURES

Organisms	Positive	Questionable	Negative
Escherichia coli			X
Klebsiella species	X		
Escherichia freundii			X
Paracolon intermedium	X		
Proteus mirabilis	X		
Staphylococcus aureus	X		
Staphylococcus epidermidis	X		
Shigella sonnei		X	
Salmonella derby (Group B)	X		
Proteus morgani	X		
Bethesda-Ballerup Group	X		
Shigella-Alkalescens-dispar	X		
Pseudomonas aeruginosa			X
Serratia species		X	
Clostridium perfringens			X
Clostridium noveyi	X		
Clostridium histolyticum			X
Streptococcus faecalis		X	

Ten separate samples over a period of months of ileostomy material from subject five were analyzed for bacteriostatic substances. These substances appeared to be of variable concentration and to be transitory in nature.

The ileostomy bacteriostatic substance was found to be ether soluble at pH 1 and 7, and positive zones of inhibition and stimulation occurred when the ether extract was used, as shown in Table 2. Fatty acids can of course be recovered in this fashion. The ether portions of the sample contained the largest amount of the inhibitory substance, as shown by the size of the zones of inhibition. Zones of inhibition were also observed against one strain of E. coli with the aqueous remainder of the extraction process, but the zones were much smaller. This was believed due to either incomplete extraction of inhibitory substance into the ether, or that the substance might have organic acid groups attached to the molecules.

A fluorescent spectrum was made on the ether extracted portion, which was a yellow, oily, and crystalline appearing substance. The absorption maxima in ether was found primarily at 305 millimicrons with emission at 382 millimicrons. Additional shoulders were also found. From this data, the literature was reviewed and substances with identical or very similar fluorescent spectra were sought.

Novobiocin, a commonly used antibiotic active against bacteria that may be inhabitants of the intestinal tract of man, was found to have a spectra of 305 millimicrons absorption maxima, and emission at 390 millimicrons. (Spector, 1957). Subject five it should be emphasized, was not receiving any medication and was on a normal diet. A pure sample of Novobiocin was obtained from the Upjohn Company, and fluorescent and infra red spectra were made. The results were similar between the ether soluble bacteriostatic extract obtained and the pure samples of novobiocin. The antibiotic was also inhibitory to the three test organisms. Both the bacteriostatic substance and Novobiocin were photolabile. Since Novobiocin is a product of Streptomyces niveus and Streptomyces spheroides (Spector, 1957) an attempt was made to isolate these actinomycetes from the ileostomy specimens that demonstrated inhibition. None however were found

TABLE 2

THE ACTION OF THE ETHER EXTRACTED INHIBITORY SUBSTANCE, ISOLATED FROM SUBJECT FIVE, ON THREE SPECIES OF BACTERIA

Indicator Organism (and source)	Ether Control	Ether Extract		Remaining Aqueous Portion of Extract	
		Inhibition	Stimulation	Inhibition	Stimulation
		pH 1			
Escherichia coli (General Hospital strain)	-	+ (21 mm)	-	X	X
Escherichia coli (A.T.C.C. 11229)		+ (16 mm)	+	X	X
Shigella sonnei (Biological Sciences, U.C.)		+ (12 mm)	+	X	X
		pH 7			
Escherichia coli (General Hospital)	-	+ (16 mm)	+	+ (10 mm)	+
Escherichia coli (A.T.C.C. 11229)		+ (16 mm)	+		
Shigella sonnei (Biological Sciences, U.C.)		+ (20 mm)			+

with the media and techniques used.

In baseline studies on subject five Proteus mirabilis and Proteus vulgaris were found to give way to Proteus morgani. No Staphylococcus aureus, Salmonella or Shigella species were found. Escherichia coli was found to disappear over the one year study, gradually being replaced by Pseudomonas species, including Pseudomonas aeruginosa.

In vitro studies of the inhibitory substance insolated from subject five however, did inhibit some strains of Shigella sonnei and Escherichia coli, but not Pseudomonas species, Proteus species, Salmonella species, Shigella species, or Staphylococcus aureus.

Another approach was now taken based on the above information to identify the possible origin of this inhibitory substance isolated from subject five. All of the pure stock cultures that were isolated originally from subject five, throughout this research study, were now tested for the presence of bacteriocin producing strains. Pure broth cultures of the isolated test strains were swabbed individually onto the surface of deep-poured trypticase soy agar plates and incubated at 37 C for four days. Plugs of agar were then cut from these plates and placed right side up, onto plates seeded with an indicator organism. The indicator strains used were the same three that were used for testing the presence of inhibitory substance in the cell-free filtrates of ileostomy material.

The results of this study indicated, as shown in Table 3, that five different test strains of Pseudomonas species isolated from the ileostomy specimens obtained from subject five could produce inhibitory substances to which these indicator strains were sensitive. These five Pseudomonas species were then tested for the production of inhibitory substances and their effect on the Escherichia coli strains, Proteus vulgaris, and Proteus mirabilis isolated in earlier studies from subject five. The results of this study are found in Table 4.

Of the 12 strains used as indicators, all Escherichia coli (all 10) strains

TABLE 3

SPECIES OF BACTERIA PRODUCING INHIBITORY SUBSTANCES, ISOLATED FROM SUBJECT FIVE, USING THREE INDICATOR STRAINS OF BACTERIA

Test Strains (code number of isolate)	Indicator Strains		
	Escherichia coli (A.T.C.C. 11229)	Escherichia coli (General Hospital)	Shigella sonnei (Biol. Sc., U.C.)
11	-	-	-
13	-	?	?
16	-	?	-
17	?	?	-
18	-	?	-
1	?	-	?
2	++	++	++
3	-	-	-
4	-	-	+
5	++	++	++
6	-	-	?
7	++	++	++
8	?	-	?
9	?	-	-
10	++	++	++
11	-	?	-
12	-	-	-
15	++	++	++

++ = large zone of inhibition

+ = small zone of inhibition

? = questionable zone of inhibition

- = no zone of inhibition

TABLE 4

THE ACTION OF FIVE SPECIES OF PSEUDOMONADS, ISOLATED FROM SUBJECT FIVE, ON VARIOUS STRAINS OF ESCHERICHIA COLI AND OTHER BACTERIA, ISOLATED FROM THE SAME SUBJECT

Indicator Strain		Pseudomonas species Test Strain (code number of isolate)				
Escherichia coli (code number of isolate)	#	2	5	7	10	15
	12	++	++	++	++	++
	16	+	+	++	+	+
	20	+	+	+	+	+
	21	+	+	+	+	+
	2	+	+	+	+	+
	6	+	+	+	+	+
	8	+	+	+	+	+
	11	++	+	++	+	+
	13	+	+	+	+	+
	17	+	+	+	+	+
Proteus vulgaris	19	-	-	-	-	-
Proteus mirabilis	1	-	-	-	-	-

\+ = zone of inhibition

++ = large zone of inhibition (clear zone greater than 10 mm in diameter)

\- = no zone of inhibition

were inhibited by all of the Pseudomonas species, but Proteus vulgaris and Proteus mirabilis were not inhibited by any.

It is of particular interest to note that Reeves in 1967 pointed out that rough strains of Escherichia coli were particularly susceptible to the action of bacteriocins produced by Pseudomonas species. Most of the strains of Escherichia coli isolated from subject five were rough. This could, therefore, be a possible mechanism accounting for the disappearance of Escherichia coli from the ileum of subject number five.

In addition it may be noted that all five of these isolated strains of Pseudomonas organisms produced a bluish-green pigment on trypticase soy agar plates. The pigment normally found in most Pseudomonas cultures consists of a mixture of products, principally pyocyanin and/or a mixture of fluorcescin. All of the older cultures in this study displayed a reddish-brown pigment which is reported to be an oxidation product of pyocyanin (Wilson and Miles, 1964).

Pseudomonas pigments are known to have antibacterial as well as lytic actions on a wide range of gram-positive as well as gram-negative organisms. Wilson and Miles (1964) reported that an oily substance isolated from cultures of Ps. aeruginosa also exists. This substance was ether extractable under acidified conditions, and is also fluorescent. It was reported to be active on various bacteria.

Therefore, it appears that the inhibitory substance or substances isolated from subject five may be a metabolic product of Pseudomonas species. Additional studies, however, must first be performed to determine its full ecological significance in the ileum of man.

Smith in 1966, reported that he isolated an unidentified, clear, pale-yellow, oily residue from the stomach of suckling rabbits. This substance was extracted from the stomach contents with chloroform and was found to have in vitro inhibitory properties active against Staphylococcus aureus, Candida albicans, Lactobacillus acidophillus, Streptococcus faecalis, and Escherichia coli. In this case, however, no attempts were made to isolate any of the

bacterial species present in the stomach contents.

It may be noted that bile acids are fluorescent (Eriksson and Sjovall, 1955-1956). The spectra of these acids are similar to the spectra found in this study of the inhibitory substance. Since bile would be expected to be present in large amounts in the ileum, bile must also be considered since it is also bactericidal to many bacterial organisms.

References

1. Ericksson, S. and Sjovall, J., 1955-1956, "The absorption spectra of bile acids in sulfuric acid. Bile acids and steriods, 31 Arkiv for Kemi. 9:303-310.

2. Reeves, P. 1965, "The bacteriocins", Bacteriol. Revs., 29: 25-45

3. Scarpino, P. V., Deters, L. E., and Niemeier, R. W., 1969, "Investigation of the types and numbers of aerobic and anaerobic bacteria occurring in intestinal specimens obtained from adult human ileostomy and colostomy subjects." Bacteriol. Proc., 1969:103

4. Smith, H. W., 1966, "The antimicrobial activity of the stomach contents of suckling rabbits." J. Path. Bacteriol. 91:1-9.

5. Spector, W. S. (ed.) 1957, Handbook of Toxicology. Vol. II, Antibiotics W. B. Saunders Co., Philadelphia, Pa., pp. 127-129, 228, 229.

6. Wilson, G. S. and Miles, A. A., 1964. Topley and Wilson's Principles of Bacteriology and Immunity. The Williams and Wilkins Co., Baltimore, Md.